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Bacterial endophytes of grapevine (*Vitis vinifera* L.) as promising tools in viticulture: isolation, characterization and detection in inoculated plants

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Abstract. Bacterial endophytes may positively influence the host plants, and the search for new strains with beneficial properties is a promising research direction. We isolated culturable endophytic bacterial strains from cuttings of grapevine of four cultivars, identified and characterized their physiological properties and studied colonization process and localization sites of the introduced DsRed-labeled strain. The taxonomic diversity of microorganisms isolated from the inner tissues of grapevine was identified based on the analysis of the 16S rRNA gene fragments. Several promising strains of endophytic bacteria were isolated. DsRed+ phenotypes were obtained by transformation. Their introduction into grapevine plants made it possible to reveal their localization in the plant vascular tissues.

Key words: grape (*Vitis vinifera* L.), bacterial endophytes, 16S rRNA gene, colonization activity, DsRed- labeling, CSLM.

INTRODUCTION

Internal tissues of plants harbour numerous bacteria, which may be involved in neutral, positive or negative interactions with the host plant (Lipka & Panstruga, 2005, Tichonovich & Provorov, 2009). Bacterial endophytes may stimulate plant growth (Taghavi et al., 2009), increase the availability of mineral nutrition elements (Malinowski et al., 2000), biologically fix nitrogen (de Bruijn et al., 1997; Doty, 2011), suppress the development of pathogenic microorganisms (Kloepper et al., 2004; Melnick et al., 2008; Bae et al., 2011) and enhance the immune response of plants (Kloepper et al., 2004; Melnick et al., 2008). Colonizing the econiches of phytopathogens, beneficial bacterial endophytes may act as biocontrol agents (Berg et al., 2005). The search for new endophytic microorganisms with beneficial properties is a promising research direction (Ryan et al., 2008; Chebotar et al., 2015).

In particular, endophytic bacteria may serve as biocontrol agents of various fungal and bacterial diseases of grapevine. The presence of endophytic bacteria from the genera *Streptomyces, Pseudomonas, Bacillus* and some others decreased the negative impact of

the phytopathogens *Fusarium oxysporum, Erysiphe necator, Plasmopara viticola* and *Xylella fastidiosa* on the grapevine plants (Compant et al., 2013).

In this work, we isolated culturable strains of bacteria from the grapevine cuttings of four cultivars and characterized their physiological properties. The main aim of this work was to identify potentially beneficial endophytic bacterial strains and study the colonization pattern of introduced strains.

MATERIALS AND METHODS

Plant material, bacterial and fungal strains, media

We isolated bacterial endophytes from plants of grapevine (*Vitis vinifera* L.) of four cultivars (Fetyaska belaya, Rkatsiteli, Muskat belyi and Muskat chernyi) sampled in Astrakhan region and Krasnodar region (Russia). Analyzed in this paper 4 grapes cultivars according to their areas of origin and distribution, as well as the aggregate of botanical, morphological and anatomical characteristics and properties related to the Euro-Asian group. According to the classification of A.M. Negrul (Negrul, 1968) this Euro-Asian cultivars by geographical distribution and biological characteristics relate to the one ecological-geographic group – the group of the Black Sea coast – *Vitis vinifera convar. pontica Negr*. The experimental period included 2 years (2014–2015) investigations, sample collection procedure was carried in the end of May in the same geographical points. Cuttings of lignified grapevine stems (30–35 cm in length) were placed into sterile polythene bags, transported to the laboratory and used for isolation of endophytic bacteria.

Fungicidal properties of the isolated strains were tested on the phytopathogenic and toxicogenic fungi *Fusarium oxysporum* (*Fusarium* wilt of grapevine), *Botrytis cinerea* (grey mold) and *Alternaria* sp. (Alternaria rot) (strains from the All Russia Research Institute for Plant Protection (VIZR) collections, kindly provided by Dr T. Yu. Gagkaeva). Antibacterial properties of the isolated strains were tested on five strains of common phytopathogenic bacteria: *Pseudomonas syringae* 213, *Erwinia carotovora var. atroseptica* 822, *Erwinia carotovora var. atroseptica* 3304, *Pseudomonas syringae pv. atrofaciens* p-88, *Xanthomonas campestris* 7604 (strains from the VIZR collections, kindly provided by Dr A.M. Lazarev).

The seeds of cress (*Lepidium sativum* L.) cultivar 'Vesenniy' and tomato (*Solanum lycopersicum* L.) cultivar 'Ataman' were used as the test objects for study of bacterial plant growth promotion (PGP) – properties, generally auxin production and colonization activity.

We used agarized and liquid media: R2A (Hycase SF – 0.5 g l^{-1}; yeast extract – 0.5 g l^{-1}; peptone – 0.5 g l^{-1}; glucose – 0.5 g l^{-1}; starch – 0.5 g l^{-1}; potassium hydrophosphate (K_2HPO_4) – 0.3 g l^{-1}; magnesium sulfate ($MgSO_4 \cdot 7H_2O$) – 0.024 g l^{-1}; sodium pyruvate ($CH_3COCOONa$) – 0.3 g l^{-1}; agar-agar – 17 g l^{-1}), 2% potato agar (potato broth (20 g of potato per 1 l of water) and agar-agar – 17 g l^{-1}), potato-dextrose agar PDA (Difco, USA), Muromtsev medium (glucose – 10 g l^{-1}; asparagine – 1 g l^{-1}; potassium sulfate – 0.2 g l^{-1}; magnesium sulfate ($MgSO_4 \cdot 7H_2O$) – 0.2 g l^{-1}; maize extract – 0.02 g l^{-1}; calcium orthophosphate ($Ca_3(PO_4)_2$) – 4.5 g l^{-1}; agar-agar – 20 g l^{-1}).

Isolation and identification of bacterial endophytes

Endophytic bacteria were isolated from grape plants using an original method of surface sterilization of plant samples. Four to five lignified grapevine cuttings with a length of 10–15 cm were weighed, placed in sterile 500 mL flask, washed three times in sterile water, purified of excessive tissue and placed for a few seconds in 70% ethanol. Then the cuttings were sterilized for 10 min in 10% hydrogen peroxide and washed five times in sterile water. Surface-sterilized cuttings were cut under sterile conditions, pieces of xylem and core were plated onto R2A medium (Difco, USA). The plates were incubated for 5 days at 20 °C, the isolates obtained by plating were purified and stored at -80 °C in sterile broth containing 20% glycerol.

Bacterial DNA was extracted from the isolated bacterial strains using lysis by lysozyme and SDS, protein precipitation by 3M sodium acetate, purification by phenol:chloroform:isoamyl (24:24:1) and DNA precipitation by isopropanol. Briefly, portions of the 16S rRNA genes were obtained via PCR amplification with primers 27 fm (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1522R (5′-AAG GAG GTG ATC CAG CCG CA-3′) (Weisburg et al., 1991). The amplified DNA fragments were digested with the two nucleases Msp I and Hae III. The resulting fragments were separated on a 2% agarose gel and the profiles of the endophytic strains were compared. For nucleotide sequence determination, PCR products were separated on a 1% agarose gel, recovered and purified from agarose using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Sequencing was performed according to the manufacturer's recommendations for GS Junior (Roshe, The Switzerland). Similarity searches in GenBank were performed using BLAST (http://www.ncbi.nlm.nih.gov/blast/; Altschul et al., 1997).

Physiological activity of bacterial endophytes

Endophytic bacteria that could produce auxins were cultured on R2A liquid nutrient medium with the addition of 500 мg l^{-1} of L-tryptophan. To identify strains producing much indole-3-acetic acid (IAA) and its derivatives, we used Salkowski reagent, which gives a characteristic pinkish-red staining with IAA (Salkowski, 1885; Glickmann & Dessaux, 1995). IAA is the main hormone of the auxin type responsible for phytostimulation.

Antagonistic activity of the isolated strains against fungal phytopathogens was tested using the well method (Magnusson & Schnurer, 2001) on PDA medium. Antagonistic activity of the isolated strains against bacterial pathogens was tested with the use of agar blocks method (Zenova et al., 2002) on 2% potato agar.

Protease, amylase and lipase activity were revealed with the use of standard techniques for assessing bacterial enzyme activity (Netrusov et al., 2005; Tepper & Shilnikova, 2005). Pectinase activity was studied in the medium with bromothymol blue as an indicator (Jakob et al., 2009). Cellulase activity was tested in the medium with microcrystalline cellulose following Kasana et al. (2008). Solubility of poorly soluble phosphorus compounds was revealed in the Muromtsev medium (Netrusov et al., 2005) with the addition of 4.5 g of $Ca_3(PO_4)_2$.

To reveal growth-stimulating effect, the strains under study were grown on liquid R2A medium up to the optical density of 1 at a wavelength of 600 nm. After that, the nutrient medium was washed off the culture with the physiological solution (0.85% NaCl solution) and the optical density was made to reach 0.5. Seeds of cress (*Lepidium*

sativum) were soaked in the suspension of bacterial cells at 1:10, 1:100 and 1:1000 dilutions for 15 min. Three days later, root length and shoot length was measured in germinated seeds. Sterile distilled water without bacterial cells was used as the control. Data on the root and the shoot length were statistically processed with the help of the one-factor dispersion analysis using DIANA software (ARRIAM, Russia).

Construction of DsRed-tagged *Pseudomonas* sp. 2ES (strain 2ES-DsRed)

Plasmid pMP4662 (Bloemberg et al., 2000) was transformed into *Pseudomonas* sp. 2ES by electroporation. Electrocompetent cells were prepared according to Choi (2006). For electroporation, 500 ng of plasmid DNA purified by gel electrophoresis was mixed with 100 µl of electrocompetent cells and the mixture was transferred to a 2 mm gap width electroporation cuvette. Immediately after applying a pulse (settings: 25 µF; 200 Ω; 2.0 kV on a Bio-Rad GenePulser; Bio-Rad), 1 ml of room temperature LB medium was added. The cells were transferred into a glass tube and shaken for 2 h at 37 °C. The cells were harvested in a microcentrifuge tube and 100 µl of the harvest was plated on an LB + Tc 20 мg l^{-1} plate. The plates were incubated at 37 °C until colonies appeared (usually within 24 h). Cells pulsed without the addition of DNA served as controls.

After transforming the plasmid to *Pseudomonas* sp. 2ES-DsRed by electroporation, the stability of the plasmids in the rhizosphere (without the antibiotic pressure) was assessed. For this, tomato seedlings were inoculated with the 2ES-dsRed derivatives as described below and their growth was studied in a gnotobiotic quartz sand system. After seven days, bacteria were isolated from the root tip as described elsewhere (Simons et al., 1996) and plated on LB agar plates without antibiotics.

Introduction and visualization of strain 2ES-DsRed within plant tissues

Bacteria harboring plasmids with DsRed genes were examined using confocal scanning laser microscope Carl Zeiss LSM 510 META NLO (Carl Zeiss, Germany). Filter sets tailored to the specific chromophores were used for DsRed, 510-nm excitation with 550–575 nm emission. Tomato roots colonized by *P.* sp. 2ES-DsRed after 5 days of growth in the gnotobiotic sand system were washed in phosphate-buffered saline (PBS) to remove sand particles and mounted in PBS on an attached coverslip. Samples were examined under the confocal scanning laser microscope.

Pseudomonas sp. 2 ES-DsRed strains were cultivated on liquid LB + Tc 20 mg l^{-1} at 28 °C and 200 rpm for 48 hours. The culture of bacteria was added to the fresh inoculum based on PNS solution to achieve the final concentration of 10^5 CFU ml^{-1}. Grape cuttings (Rkatsiteli cultivar) were placed in cylindrical glass bottles filled with PNS solution (1/5 of the vessel volume) containing the inoculum of *Pseudomonas* sp. 2ES-DsRed (Fig. 1, B). The cuttings were kept in the bottles with the inoculum in a climate chamber at a temperature of 20 °C for 7 days. Sections with a thickness of 70–80 µm were made using the vibratome and examined under the confocal scanning laser microscope.

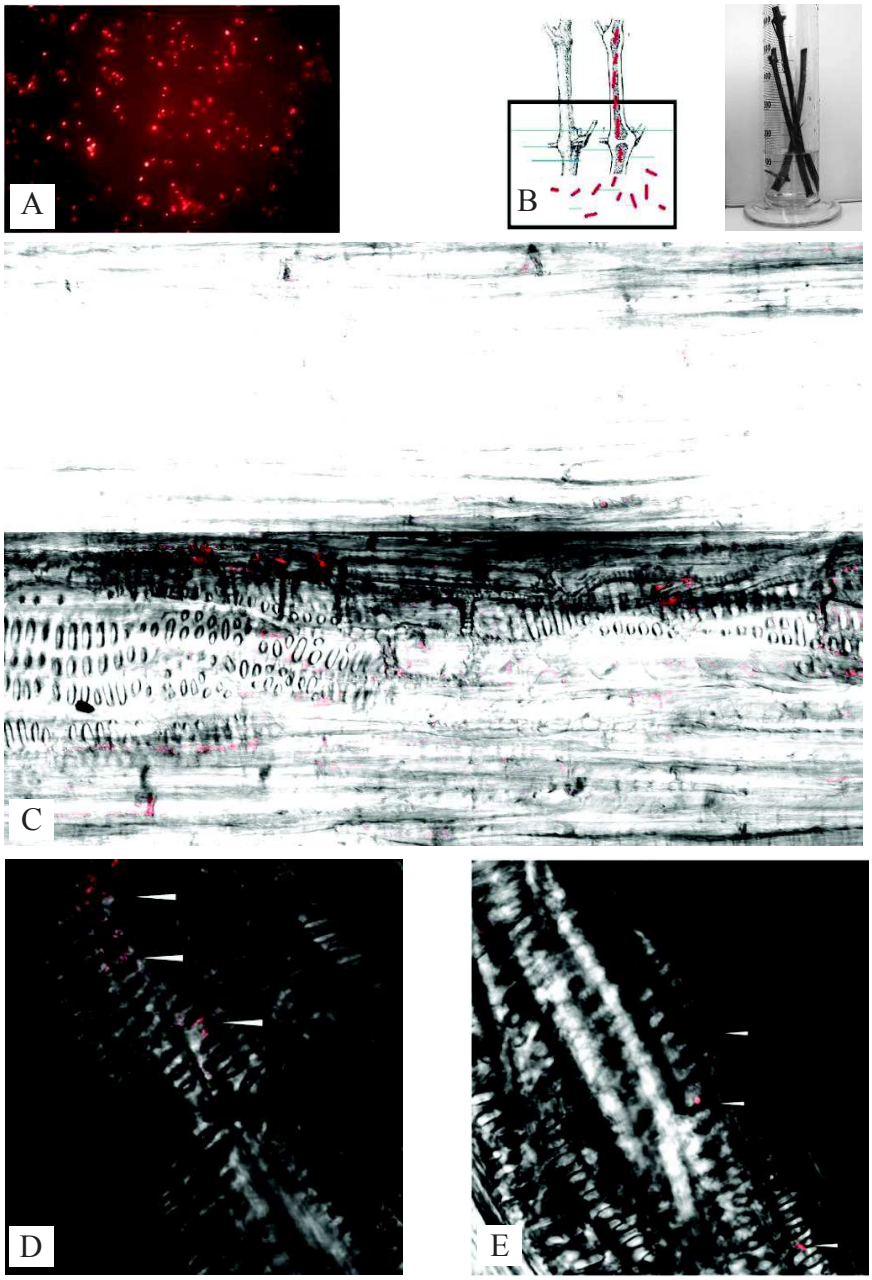

Figure 1. Introduction and localization of DsRed- labeled strain *Pseudomonas sp.* 2ES+DsRed in grapevine stems: A – Fluorescence of the cells of the transformed strain *Pseudomonas sp*. 2ES+DsRed. Epiluminescent microscopy. 1000X. B – Scheme of introduction of the biocontrol strain *Pseudomonas sp*. 2ES+DsRed; C – The main area of localization of the introduced strain *Pseudomonas sp.* 2ES+DsRed in pitted vessels of the grapevine vascular tissue; D, E – microcolonies and single cells of *Pseudomonas sp.* 2ES+DsRed in pitted vessels of grapevine. Bacterial cells were detected as a red signal (arrows). Confocal scanning laser microscopy with detection of DsRed emission in the wavelength of 550–575 nm, magnification 400X, 200X.

RESULTS

We isolated more than 40 culturable bacterial strains from the tissues of grapevine plants belonging to of four cultivars and characterized their morphological and cultural features and cultivation requirements. Most of the isolated bacteria (> 80%) were Gram-positive. They were represented by very small (about 1.5–2.0 μm) rounded or elliptic cells to. On the R2A medium they formed dry flat colonies with a scalloped edge. The colonies were opaque and beige or milky whitein color and could be fast or slowly growing.

The taxonomic diversity of the microorganisms isolated from the inner tissues of grapevine (four cultivars) was identified at the molecularly with level based on the analysis of the 16S rRNA gene fragments. A characteristic feature of bacterial populations of grapevine of different cultivars and different geographical origin was the presence of the genus *Bacillus* (Table 1), which formed characteristic bacillar colonies on the media. Species of this genera may be specific of the endophytic bacterial community of grapevine (Baldan et al., 2016).

Most of the isolated strains showed a complex activity against the test cultures of phytopathogens. The highest antibacterial and antifungal activity was shown by *Bacillus amyloliquefaciens* and *B. atrophaeus*, which suppressed most of the test cultures. *B. subtilis*, *B. pumilus, B. megaterium* and *Pantoea vagans* were moderately active against phytopathogens. Several strains with a complex biocontrol activity – namely *B. atrophaeus* SP14, *B. subtilis* Sof4, *B. amyloliquefaciens SP2,* and *B. amyloliquefaciens SP16* – were chosen as promising candidates for the development of phytoprotective biopreparations to be used in viticulture.

Five isolates (*Pantoea vagans* Sof1, *B. megaterium* Sof3, *B. megaterium* Sof5, *B. thuringiensis SP11,* and *Pseudomonas sp.* 2ES) showed plant growth promoting activity. These strains synthesized auxins on the medium with L-tryptophan and stimulated the development of cress seedlings, increasing the length of roots by 27–79% as compared with non-inoculated plants. These bacterial strains had a rather hydrolytic enzyme activity. Strains actively releasing proteinases, amylases, lipases, pectinases and cellulases were identified.

Transformation of several promising strains of endophytic bacteria belonging to the genus *Pseudomonas* (chosen due to the complex of physiological and beneficial properties) allowed to obtain DsRed+ phenotypes. We chose the transformant of strain *Ps. sp*. 2ES+DsRED carrying the plasmid pMP4662, which was as good as the initial native strain in respect of fungicidal, growth-stimulating and colonization properties (Table 2). The cells of this transformant showed a bright fluorescence (maximum excitation at a wavelength of 554 nm, maximum emission at 586 nm). The image of a pure culture of the obtained transformant of *Pseudomonas sp.* 2ES+DsRED under an epiluminescent microscope is shown in Fig. 1A.

The introduction of the marked strain *Pseudomonas sp*. 2ES+DsRED into the vegetative parts of grapevine plants made it possible to reveal its localization in the vascular tissue (Fig. 1, C, D, E). *Pseudomonas sp*. 2ES+DsRED were localized in pitted vessels of grapevine shoots, as single cells or in small groups. The strain was mobile and could circulate in the vessels.

Table 1. Taxonomic position, beneficial properties and physiological characteristics of the most promising strains of endophytic bacteria associated with grapevine

Grape cultivar	Strain	Fungicidal activity	Bactericidal activity	Production of auxins, on L-tryptophane containing medium	Enzymatic activity	Ability to dissolve phosphates ($Ca_3(PO4)_2$)	Plant growth promoting activity
Black Muskat	*Pantoea vagans* Sof1	Al	Pst,	+	Pc	-	+79%
	Bacillus sp. Sof2	-	Pst,Psa,Clm	-	-	-	-
	B. megaterium Sof3	-	Pst, Psa	+	A,L	+	+69%
	B. pumilus SP7	Fo	-	-	P	+	-
	B. atrophaeus SP13	Al,Bc,Fo	Pst	-	A,C,L,P,Pc	+	-
	B. atrophaeus SP14	Al,Bc,Fo	Pst, Ec	-	A,C,L,P,Pc	+	-
	B. subtilis SP15	Al,Bc,Fo	Ec, Xc	-	A,C,L,P,Pc	+	-
Fetyaska	*B. subtilis* Sof4	Al,Bc,Fo	Pst, Clm	-	A,P	-	-
	B. megaterium Sof5	-	Pst	+	-	-	+61%
	B. subtilis SP1	Al,Bc,Fo	Pst,Psa,Ec,Xc	-	A,C,L	+	-
	B. amyloliquefaciens SP2	Al,Bc,Fo	Pst,Psa,Ec,Xc	-	A,C,L	+	-
	B. amyloliquefaciens SP3	Al,Bc,Fo	Pst,Psa,Ec,Xc	-	A,C,L	+	-
	B. amyloliquefaciens SP4	Al,Bc,Fo	Pst,Ec,Xc	-	A,C,L,P	+	-
	B. amyloliquefaciens SP5	Bc,Fo	Pst,Psa,Ec,Xc	-	A,C,L,P	+	-
	B. amyloliquefaciens SP6	Bc,Fo	Pst,Psa,Ec,Xc	-	A,C,L,P	+	-
	B. amyloliquefaciens SP8	Al,Bc,Fo	- Pst,Psa,Ec,Xc	-	A,C,L,P	+	-
	B. pumilus SP9	Fo	-	-	P	+	-
	B. pumilus SP10	Bc,Fo	-	-	P,Pc	+	-
	B. thuringiensis SP11	Bc,Fo	-	+	A,C,L,P	+	+38%
	B. pumilus SP12	Al,Bc,Fo	-	-	A,P	+	-
	B. amyloliquefaciens SP16	Al,Bc,Fo	Pst,Psa,Ec,Xc	-	A,C,L,P	+	-
	B. subtilis SP17		Ec	-	A,C,L,P	+	-
Rkatsiteli	*Pantoea vagans* Sof6	Al,Bc,Fo	Pst, Psa, Ec,	-	A,L,P	-	-
	Pseudomonas sp. 2ES	Al,Bc,Fo	-	+	A,P	-	+27%
	B. amyloliquefaciens SP18	Al,Bc,Fo	Pst,Ec,Xc	-	A,C,L	+	-
	B. amyloliquefaciens SP19	Bc,Fo	Pst,Ec,Xc	-	A,C,L	+	-
	B. amyloliquefaciens SP20	Bc,Fo	Pst,Ec,Xc	-	A,C,L	+	-
	B. amyloliquefaciens SP21	Bc,Fo	Pst,Ec,Xc	-	A,C,L,P	+	-
Muskat	*B. amyloliquefaciens* 22	Bc,Fo	Pst,Psa,Ec,Xc	-	A,C,L,P	-	-

Note: Fungicidal activity against: Al – *Alternaria sp*., Bc – *Botrytis cinerea*, Fo – *F. oxysporum*; bactericidal activity against: Pst – *Pseudomonas syringae pv. tomato*, Psa – *P. syringae pv. atrofaciens*, Ec – *Erwinia carotovora pv. atroseptica*, Xc – *Xanthomonas campestris*, Clm – *Clavibacter michiganensis pv. sepedonicum*; enzymatic activity: A – amylase, C – cellulose, L – lipase, P – protease, Pc – pectinase; Plant growth promoting activity – % increment of root length of radish seedlings.

Table 2. Comparative characteristic of physiological-biochemical properties of the genetically modified strain *Pseudomonas. sp*. 2ES+DsRed and the native form

Characteristic	*Pseudomonas sp*. 2ES+DsRed	Native strain *Pseudomonas sp*. 2ES
Number of bacteria in 24 h culture on LB medium, Cfu ml^{-1}	$750 \cdot 10^6$	$775 \cdot 10^6$
Zone of inhibiting growth of *F. culmorum* (5 day), mm	23	24
Zone of inhibiting growth of *F. oxysporum* (5 day), mm	26	25
Production of auxins, on L-tryptophane containing medium	+	+
Number of bacteria in rhizosphere of tomato plants, Cfu g^{-1} rhizosphere substrate	$250 \pm 25 \cdot 10^4$	$137 \pm 43 \cdot 10^4$
Number of bacteria on the roots of tomato plants, Cfu cm^{-1} root length	$37 \pm 5 \cdot 10^4$	$45 \pm 8 \cdot 10^4$

DISCUSSION

In this work about 40 strains of culturable bacteria were isolated from the endosphere of grapevine. Gram-positive spore-forming bacteria from the genus *Bacillus* dominated among the isolated strains. Their abundance made up 89.6%.

Numerous previous studies have shown that both Gram-positive and Gram-negative bacteria can be present in the endosphere of grapevine. For instance, strains of *Bacillus spp., Pseudomonas spp. and Burkholderia spp*. were isolated from leaves and shoots of grapevine (West et al., 2010). Numerous and diverse species of *Bacillus, Pseudomonas* and *Pantoea* were isolated from grapevine flowers, berries and seeds (Compant et al., 2011), with the colonization niches visualized by FISH method.

Taxonomic diversity of bacterial endophytes of grapevine plants was previously studied using culture independent technique (detection by LH-PCR) (Bulgari et al., 2014) and bacteria previously reported as biocontrol agents (*Burkholderia, Methylobacterium, Sphingomonas* and *Pantoea*) were identified. Similar taxa have also been found with the use of ARISA fingerprinting and pyrosequencing of 16S rDNA (Campisano et al., 2014) and high levels of the dominant genera (*Ralstonia*, *Burkholderia* and *Pseudomonas*) were detected in all the samples. Bacterial endophytes from genera *Bacillus, Pseudomonas* and *Pantoea* have also been found in the internal tissues of grapevine plants (Andreolli et al., 2016). Antifungal activity analysis showed that two of the *Bacillus* strains possess growth antagonistic effect against all the tested fungal strains.

In this work, we screened bacterial endophytes of grapevine for economically valuable properties such as antagonism against fungal and bacterial phytopathogens, ability to produce IAA, mineralization of poorly soluble phosphorus compounds, enzymatic activity and the ability to stimulate plant growth. As a result, we chose a number of strains with a complex of beneficial properties: *B. amyloliquifaciens*, *B. atropheus*, *B. subtilis* and *B. megaterium* which are also described as grape endophytes by some authors (Huang et al., 2011; Liu et al., 2014; Wang & Liang, 2014; Wu et al., 2015).

The strain *B. thuringiensis* SP11 is of special interest. Its introduction into grapevine plants might make it possible to control insect pests (Monnerat et al., 2009; Tanuja et al., 2013; Tao et al., 2014) by purely biological means without the genetic modification of plants with the use of Bt genes.

Gram-negative strains, in particular, those from the genus *Pseudomonas*, are also promising in respect of PGP-properties. These common microorganisms might be good biocontrol and growth-stimulating endophytic agents of grapevine (Kilani-Feki et al., 2010; Verhagen, 2010).

In our study, we showed that an introduced DsRed-markered strain with biocontrol and growth-stimulating properties may successfully colonize pitted vessels (xylem) of grapevine shoots. While the localization of native strains in the leaves (Lo Piccolo et al., 2010) and berries (Compant et al., 2011) has been shown before, the introduced strain was visualized in the xylem of grapevine for the first time.

Endophytic PGP bacterial strains maybe used to colonize the vascular tissues of grapevine cuttings before rooting. These strains may biocontrol phytopathogens and stimulate plant growth during rooting and further development. In this way, self-sufficient plant-microbial systems protected from bacterial and fungal infections would arise. The future studies will include the investigation of colonization strategy by native (not modified) bacillar strains in the inner tissues of grapes cuttings with FISH methods. Also we plan to study the taxonomy of bacterial endophytes from Euro-Asian grape cultivars using metagenome analysis of bacterial community.

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